(2)

Studies on Activation and Inhibition of Phospholipase D in Small Unilamellar Vesicles

A Thesis Submitted to the Graduate School of Pharmaceutical Sciences of Kyoto University

by Izumi Yamamoto

Contents

Abbreviations

PLD	phospholipase D from *Streptomyces chromofuscus*
SUV	small unilamellar vesicles
PC	egg yolk phosphatidylcholine
LPC	egg yolk lysophosphatidylcholine
PA	egg yolk phosphatidic acid
DAG	1,2 diacylgylcerol
Chol	cholesterol
Toc	DL- α- tocopherol
HED	heptaethyleneglycol dodecylether

Preface

Phospholipase D (E.C.3.1.4.4.) cleaves the distal phosphodiester bond of phospholipids, generating PA and a free base [1]. The main substrate for the enzyme is phosphatidylcholine [2], and the enzyme activity is more dependent on structure of phosphatidylcholine derivative than on source of the enzyme [3]. Phospholipase D activities, observed in both membrane [4] and cytosolic [5] fractions of mammalian cells, play key roles in membrane trafficking and regulation of mitosis as well as signal transduction [6-9]. The lipophilic cleavage product, phosphatidic acid, is a second messenger and has activation roles in a wide variety of cells, similarly to lysophosphatidylcholine, the degradation product of phospholipase A_2 [10 - 14].

Phospholipase D is also present in plants and various microorganisms. Many of these enzymes have been purified and well characterized kinetically. Bacterial phospholipase D enzymes may be reasonable models for the mammalian isozymes. Iwasaki *et al.* [15] have cloned the phospholipase D enzyme from *Streptomyces antibioticus* in *Escherichia coli*, but, its precise regulatory mechanism remains to be clarified. Phospholipase D from *Streptomyces chromofuscus* (PLD) is a water - soluble enzyme purified from the culture supernatant [16], and mimics the effects of the endogenous enzyme in mammalian cells [17 - 19]. For example, exogenous PLD has a similar activity to endogenous phospholipase D in ovarian granulosa cell culture[18]. Exogenous addition of PLD to the medium of vascular smooth muscle cells induces marked DNA synthesis with formation of choline and phosphatidic acid [19].

The mechanism of enzyme reactions in heterogeneous interfacial systems such as membranes is not clear because of the complicated physicochemical properties of enzyme and/or substrate and interaction between them.

In this study, the dependence of PLD activity on Ca^{2+} concentration and physicochemical properties of the substrate, egg yolk phosphatidylcholine (PC), was investigated in small unilamellar vesicles (SUV) as models of

biological membranes. Furthermore, the enhanced activity of PLD bound to the SUV membrane will be discussed as compared to that of free enzyme in aqueous phase.

Chapter 1
Activation of Phospholipase D by Ca^{2+} in Small Unilamellar Vesicles

Ca^{2+} is an obligatory cofactor for interfacial catalysis by phospholipase A_2 and C from virtually all sources. The role of Ca^{2+} as a cofactor of phospholipase A_2 has been explained as polarization of the carbonyl group of the sn-2 ester linkage of PC. Activation of phospholipase D in Ca^{2+}-permeabilized HL60 cells requires the ion at micromolar concentrations in medium [22,23]. The regulation of phospholipase D activity by Ca^{2+} is important in the physiological function of the enzyme.

In addition to the hydrolytic activity, phospholipase D also catalyzes transphosphatidylation reactions in the presence of a high concentration of primary alcohol [20, 21]. This reaction has been used to monitor the presence of PLD activity for products in the millimolar concentration range in a variety of cells. pH - stat assay is also used but is not useful for detection of the lipophilic product, phosphatidic acid, in SUV membranes.

An analytical method for the PLD hydrolysis product, choline, at micromolar concentrations was developed by use of a choline electrode. This chapter describes availability of the method and the dependence of binding affinity and catalytic activity of PLD in SUV on the Ca^{2+} concentration.

Materials and Methods

Materials Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Co.(Tokyo). The purity (over 99%) was determined by thin layer chromatography (TLC) (Iatroscan Analyzer MK-5 from Iatron Laboratories, Tokyo, solvent: chloroform / methanol (3:1, v/v)). Egg yolk lysophosphatidylcholine (LPC) was purchased from Sigma Chemical Co. (St. Louis, MO).

Phospholipase D (E.C.3.1.4.4.) from *Streptomyces chromofuscus* (PLD) was purchased from Sigma Chemical Co. The specimen yielded two bands on SDS-polyacrylamide gel electrophoresis (molecular weights: 56

and 42 kDa). The molecular weight of 56 kDa agreed with the reported value for PLD [16]. The peptide of 42 kDa was separated from SUV - associating peptides by ultrafiltration in the presence of 10mM PC-SUV and 50 μM Ca^{2+}. It was concluded that the peptide of 42kDa did not associate with SUV. No choline production was detected following injection of the peptide into PC-SUV (PC: 8mM) containing 50 μM Ca^{2+}, suggesting that this peptide has no PLD activity. Furthermore, augmentation of the peptide in the specimen did not affect the enzyme activity (data not shown). The enzyme was dissolved in a buffer consisting of 10 mM Tris-HCl and 150 mM NaCl (pH 8.0), and the solution was centrifuged (10,000 rpm) at 4°C for 30 min. Three - milliliter aliquots of the supernatant were stored at 5°C. The PLD activity in each aliquot was checked by measuring the enzymatic choline production in a standard LPC solution before use. Decrease in the activity was less than 10 % after storage for one month. Choline oxidase (E.C.1.1.3.17) from *Alcaligenes* sp. was obtained from TOYOBO Co., Ltd. (Osaka).

Preparation of Vesicles and Micellar Solution PC was solved in chloroform, and the solvent was evaporated under reduced pressure. After drying under vacuum overnight, the lipid film was hydrated with Tris-HCl buffer containing 10mM Tris, 150mM NaCl and the desired amount of $CaCl_2$ or 1mM EDTA. Calcium ion concentration in the buffer was determined by EDTA titration with fra-2 as an indicator[24]. The lipid dispersion was vortexed and sonicated for 40 min under a nitrogen stream at 4°C. The probe-type sonicator used was a UD-200 from Tomy Seiko (Tokyo). The vesicle suspension (small unilamellar vesicles, SUV) was centrifuged at 3000 rpm for 10 min to remove titanium dust and stored at 25°C. No free fatty acids or phosphatidic acids resulting from the decomposition of PC during sonication were detected by TLC. Quasi elastic dynamic light scattering measurements (Otsuka Electronics, Photal LPA - 3000 / 3100) and electron microscopy of the vesicles showed a weight-averaged diameter of 30 ±15 nm, typical of small unilamellar vesicles. LPC was dissolved in chloroform / methanol (2 / 1, v / v). After

complete removal of the solvent, the residue was dissolved in Tris-HCl buffer to give a micellar solution. Phospholipid in SUV and micellar solution was assayed by the method of Bartlett [25].

Assay of PLD Activity The principle of the choline assay has been described elsewhere [26,27]. Choline produced following PC hydrolysis by PLD is oxidized to betaine by the choline oxidase with oxygen consumption. The oxygen consumed was monitored using a Clark oxygen electrode.

$$\mathbf{PC + H_2O \xrightarrow{PLD} choline + phosphatidic\ acid}$$

$$\mathbf{choline + 2O_2 \xrightarrow{choline\ oxidase} betaine + 2H_2O_2}$$

Choline oxidase was immobilized in a cellulose triacetate membrane by the method of Campanella et al. [28]. The choline oxidase membrane was mounted on top of the Clark oxygen electrode (HORIBA Ltd., Kyoto) and covered with cellophane film and O-rings. The choline electrode was stored in 0.1M glycine buffer (pH 9.0) at 5°C, and calibrated with standard choline chloride solutions before use. The enzymatic reaction was started by injection of a given volume of PLD solution into a 50 *ml* sample solution. The PLD activity was assayed within 10 hr after sample preparation.

Ultrafiltration PLD was incubated with SUV in Tris-HCl buffer. Free PLD in the incubation medium was separated from SUV-associated PLD by ultrafiltration. The ultrafiltration membrane used was rated at 100,000 MW cutoff (YM100 DIAFLO ultrafilters, W.R.Grace & Co.-Conn.). The operating pressure was about 10 psi. The free PLD amount in the filtrate was determined by measurement of the enzyme activity in a standard LPC micellar solution.

Results

Assay of PLD Activity PLD activity was measured by monitoring the concentration of choline produced using a choline oxidase - oxygen electrode at 30°C. Calibration curves on addition of micromolar concentrations of choline to a Tris-HCl buffer solution are shown in Fig. 1-1. Oxygen consumption in the reaction medium was dependent on the choline concentration, and not the rate of choline addition. It is clear that the choline electrode is useful to detect PC hydrolysis by PLD for products in the micromolar concentration range.

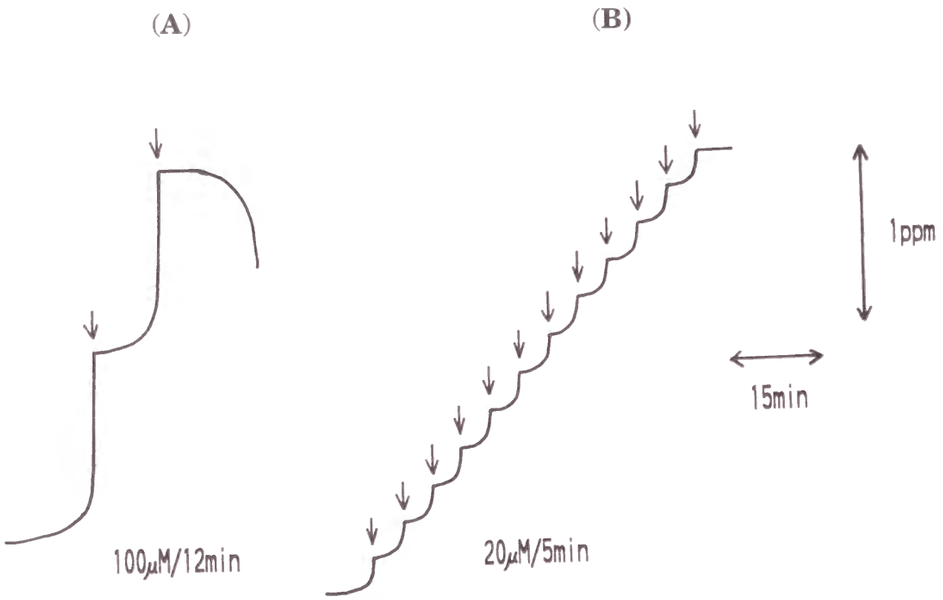


Fig. 1-1 : Choline electrode output changes of by addition of choline chloride into a Tris- HCl buffer (A) ; 100 μM portions, (B) ; 20 μM portions

Calcium Ion - Dependent Hydrolysis of PC by PLD The enzymatic hydrolysis of PC by PLD was influenced by Ca^{2+} concentration in the medium. Fig.1-2 shows the time courses of choline production in PC SUV

by PLD at various Ca^{2+} concentrations. At 7.8 μM Ca^{2+}, the initial velocity, v, of choline production was 2.34 μM / min where PC SUV concentration, S, was 2mM. The initial velocity increased with Ca^{2+} concentration in medium and was 6.89 μM / min at 50 μM Ca^{2+}. No choline production was detected in the buffer containing 1mM EDTA. Ultrafiltration experiments showed that PLD was not bound to SUV in Ca^{2+}- free medium. The double reciprocal plots, $1/v$ vs. $1/S$ (Lineweaver-Burk plots) were all linear (data not shown). Figs. 1-3A and 1-3B show the effects of Ca^{2+} on the kinetic parameters, the reciprocal of the apparent Michaelis constant, 1 / $K_{m(app)}$, and the apparent maximum velocity, $V_{max(app)}$, respectively. The kinetic parameters increased with Ca^{2+} concentration in medium. The solid lines in the figures were calculated on the basis of the reaction mechanism proposed in the Discussion.

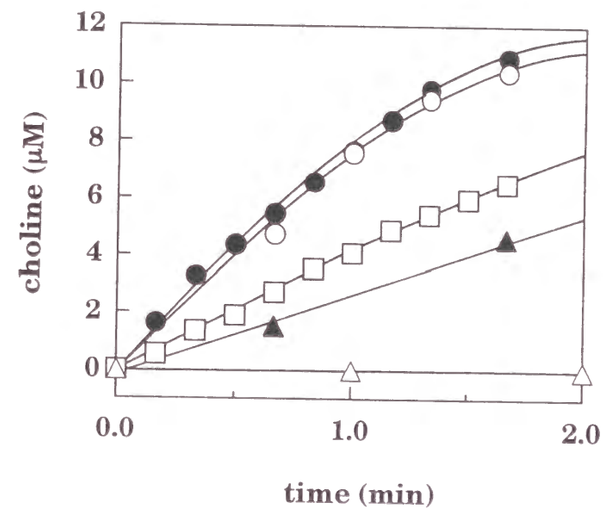


Fig. 1-2 Effect of Ca^{2+} concentration on the choline production following hydrolysis of PC by PLD. PC-SUV (2 mM) was prepared in a buffer containing 10 mM Tris, 150 mM NaCl (pH 8) and the indicated $CaCl_2$ or 1 mM EDTA. △: 1mM EDTA. Ca^{2+} concentration (μM) : ▲,7.8; □, 15; ○, 23 ; ●, 50.

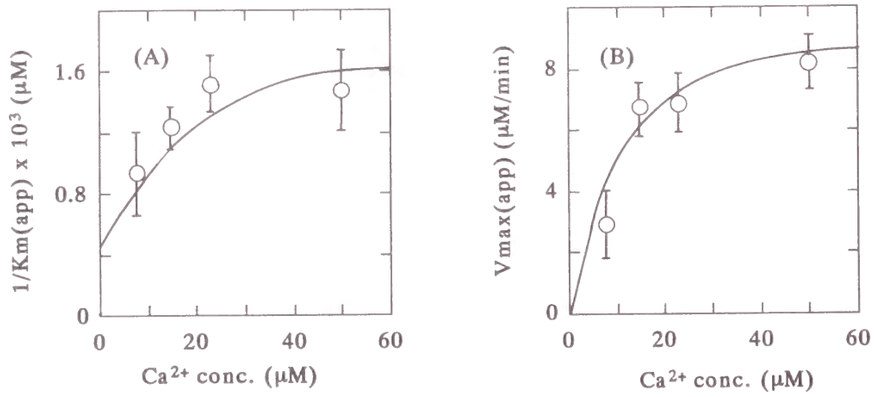


Fig. 1-3 Dependence of 1/Km(app) (A) and Vmax(app) (B) values on the Ca^{2+} concentration. Results are expressed as means ± S.E.

Discussion

Regulation of PLD Activity by Calcium Ions. PLD was associated with PC SUV in a Ca^{2+} - dependent fashion in Tris - HCl buffer. The Ca^{2+} - dependent association was similar to that of cytosolic phospholipase A_2 ($cPLA_2$), which translocates to synaptosomal [29] and phosphatidylcholine [30] membranes in a Ca^{2+} - dependent manner. When PLD was preincubated with 1mM EDTA and an aliquot was injected to a PC SUV solution containing 100μM $CaCl_2$, the choline production started after a lag time of about 2min. On the other hand, when PLD was preincubated in 23 μM $CaCl_2$ hydrolysis began immediately (data not shown). Ultrafiltration experiments showed that Ca^{2+} was essential for the PLD-binding to PC SUV. These results suggested that the binding of Ca^{2+} to PLD is followed by a conformational change of PLD, and then the enzyme likely associates with PC- SUV. Without discussion of the details of the interactions between PLD and Ca^{2+}, the hydrolysis reaction was started with PLD preincubated in buffer containing Ca^{2+} in the present study. Calcium ions increased both binding ability of PLD to SUV (1/Km(app)) and catalytic activity (Vmax(app)) (Fig. 1-3), and the maximal values of these parameters were obtained at about 20 μM Ca^{2+}. The Ca^{2+} requirement was similar to those for activation of endogenous phospholipase D in HL60

cells [22,23] and of $cPLA_2$ in a human monocytic cell lines[31] and in rabbit platelet cytosol [32]. Free Ca^{2+} concentration is about 10^{-7} M in the cytosol, and cell activation is accompanied by an increase to 10^{-6} - 10^{-4} M due to influx of extracellular Ca^{2+} or intracellular release of Ca^{2+} [33]. The Ca^{2+}-dependence of PLD suggested that the translocation and the catalytic activation may be regulated by physiological alternation of cytosolic Ca^{2+} level. However, we do not know if these results with bacterial phospholipase D (PLD) are relevant to the physiological roles of cytosolic phospholipase D in mammalian cells.

Kinetic Parameters of PC Hydrolysis. PC hydrolysis in a heterogeneous interfacial system (SUV) proceeds as shown in Fig. 1-4. Enzymes in the aqueous phase interact with the substrate molecules on the surface of the SUV membrane through either, or both mechanisms shown in Fig. 1-4. Ca^{2+} ions are essential for the PLD activity as described previously, and the substrate molecules of the enzyme are organized in the SUV membranes.

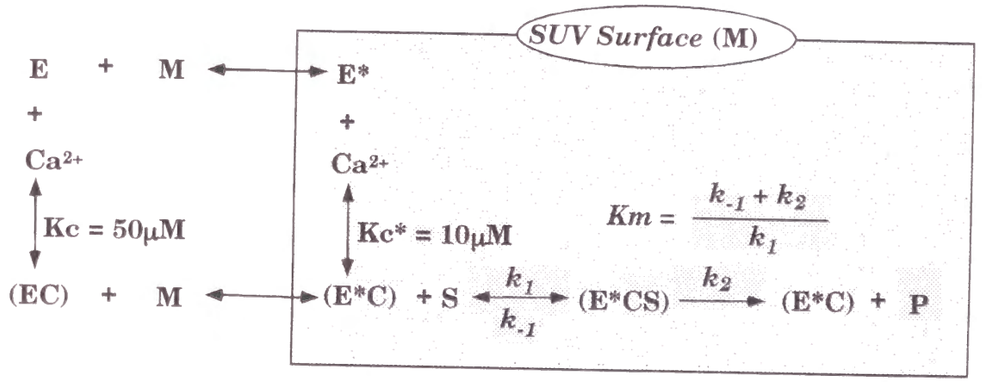


Fig. 1-4 PC hydrolysis in a heterogeneous interfacial system

E; free PLD in the aqueous phase,

E*; PLD associated with the membrane surface , **M**; membrane surface,

EC; complex of PLD and Ca^{2+} in the aqueous phase,

E*C; complex of PLD and Ca^{2+} on the membrane surface

E*CS; complex of PLD, Ca^{2+} and substrate, PC, on the membrane surface

Enzymes that interact with both Ca^{2+} and the membrane surface may hydrolyze the substrate, PC. Here, E, M, C and E* are the free PLD, the membrane surface, Ca^{2+}, and PLD associated with the SUV surface, respectively.

The concentration of choline produced at early stages was proportional to reaction time (Figs. 1-2). These results suggested establishment of the steady-state for enzyme-substrate complexes. The steady-state assumption for (E*C) and (E*CS) leads to the reaction velocity, $\boldsymbol{v}$:

$$v = V\max(app) \cdot S/(Km(app) + S) \qquad (1\text{-}1)$$

Here, Vmax(app) and Km(app) are dependent upon Ca^{2+} concentration in the reaction medium as

$$V\max(app) = \frac{V\max(app) \propto \left[Ca^{2+}\right]}{Kc^{*} + \left[Ca^{2+}\right]} \qquad (1\text{-}2)$$

$$Km(app) = \frac{Km(app) \propto \left(Kc + \left[Ca^{2+}\right]\right)}{Kc^{*} + \left[Ca^{2+}\right]} \qquad (1\text{-}3)$$

Kc and Kc* are apparent dissociation constants of PLD with Ca^{2+} in medium and on the surface of SUV membrane, respectively.

Solid lines in Fig. 1-3 are theoretical curves given by fitting the experimental data to these equations. These curves gave the dissociation constants Kc = 50 μM, Kc* = 10 μM, respectively. The Kc value agreed with that recently reported by Gong et al. [34]. These results indicated that binding of enzymes and Ca^{2+} on the membrane surface plays important roles in PLD activation.

Vmax(app) in eq. (1-2) and Km(app) in eq. (1-3) are represented as

$$V\max(app) = \frac{E_0 \cdot k_2 \cdot \left[Ca^{2+}\right]}{Km \cdot Kc' + (Km+1) \cdot \left[Ca^{2+}\right]} = f \cdot E_0 \cdot k_2 \qquad (1\text{-}2')$$

$$f = \frac{(E^{*}CS)}{(E^{*}) + (E^{*}C) + (E^{*}CS)} = \frac{\left[Ca^{2+}\right]}{Km \cdot Kc' + (Km+1) \cdot \left[Ca^{2+}\right]} \qquad (1\text{-}4)$$

$$Kc^{*} = \frac{Km}{Km+1} Kc' \qquad (1\text{-}5)$$

$$Km(app) = \frac{\{(E) + (EC)\} \cdot (M)}{(E^{*}) + (E^{*}C) + (E^{*}CS)} \qquad (1\text{-}3')$$

Here, E_o and Kc' are the total PLD concentration and the true dissociation constant of PLD with Ca^{2+} on the SUV membrane surface, respectively. f is the fraction of activated complex in the total bound PLD. The f value is independent of the SUV concentration, but depends on Ca^{2+} concentration in the aqueous phase as shown in Fig. 1-3B and possibly upon the lipid composition of SUV. A similar correlation was reported previously for the reaction of phospholipase A_2 in PC SUVs [35]. Equation (1-3') shows that K_m(app) is the apparent dissociation constant of the PLD SUV complex in the heterogeneous interfacial system. The complex enzyme reaction presented in Fig. 1-4 thus gives the rather simple equation eq. (1-1). The kinetic parameters, however, depend on the relative value of [Ca^{2+}] to Kc*.

Chapter 2
Product - Inhibition of PLD - Hydrolysis of PC and Relaxation of the Inhibition by Neutral Lipids

Neutral lipids regulate activities of enzymes in membranes by changing the membrane fluidity. For example, cholesterol (Chol) is a ubiquitous component of eukaryotic cell membranes. Physicochemical studies have suggested cholesterol - dependent modulation of membrane fluidity as a mechanism for regulating the activity of other membrane - associated components [36].

The effect of 1,2 diacylgylcerol (DAG) on bilayer membrane structures is also of interest because of their role in signal transduction in cells. DAG enhances the hydrolysis by phospholipases A_2, C and D [37 - 40]. DAG are also presumed to be potent inducers of phospholipase D in HL-60 granulocytes [39] and NG 108-15 neuroblastoma X glioma hybrid cells [40].

α-Tocopherol (Toc) is located predominantly in cellular and subcellular membranes. Recent studies have focused upon the effects of Toc on structural and dynamic properties of membranes because Toc has several physiological effects in addition to its antioxidant effects. Deficiency of Toc in animal cells changes the fluidity of membrane lipids [41] and activities of membrane-bound enzymes [42, 43]. Toc also changes the permeability and fluidity of phospholipid bilayers[44 - 48].

This chapter will deal with the effects of Chol, DAG and Toc on the activity of PLD in PC bilayers, and the correlation between enhanced catalytic activity of PLD and modification of the bilayer surface structure induced by neutral lipids. Modifications were estimated by infrared measurements of the PO_2^- antisymmetric stretching bands of PC and LPC.

Materials and Methods

Materials. PC was kindly provided by Asahi Kasei Co (Tokyo). The purity (over 99%) was determined by TLC. Cholesterol (Chol) purchased from Sigma Chemicals Co. (St. Louis, MO) showed a single spot on TLC (solvent: chloroform / acetone / methanol / acetic acid / water = 45 / 20 / 12 / 10 / 5). 1,2-Diacylglycerol derived from egg yolk phosphatidylcholine (DAG) was obtained from Serdary Research Laboratories, Inc. (London, Canada). The purity was determined to be 94 % by TLC (solvent: heptane / isopropyl ether / acetic acid (60 / 40 / 4, v/v)). The small amount of impurity (6%) was identified as the 1,3-isomer. DL- α- tocopherol (Toc) was purchased from Nacalai Tesque (Kyoto, Japan) and samples showed a single spot on TLC [solvent: cyclohexane / chloroform (2 / 1, v/v). Egg yolk lysophosphatidylcholine (LPC) and the nonionic surfactant hepta-ethyleneglycol dodecylether (HED) were purchased from Sigma Chemicals Co. (St. Louis, MO), and Nikko Chemicals, Co. Ltd. (Tokyo), respectively.

PLD was purchased from Sigma Chemicals Co. Choline oxidase (E.C.1.1.3.17.) from *Alcaligenes* sp. was obtained from TOYOBO Co., Ltd. (Osaka).

Preparation of Vesicles and Mixed Micelles. DAG, Chol, Toc and PC were mixed in chloroform, and the solvent was evaporated under reduced pressure. After drying in vacuum overnight, the lipid film was hydrated with Tris-HCl buffer, vortexed and sonicated for 40 min under a nitrogen stream at 4°C. The probe-type sonicator used was a UD-200 from Tomy Seiko Company Ltd. The vesicle suspension (small unilamellar vesicles, SUV) was centrifuged at 3,000 rpm for 10 min to remove titanium dust and stored at 25°C. Dynamic light scattering (DLS) of PC, PC / DAG, PC / Chol, and PC / Toc -SUVs was measured with a Photal LPA-3000 / 3100. The weight-averaged vesicle size of each sample was 30 ± 15 nm. The effects of mixing of neutral lipids with PC on the vesicle size were small.

Lipid (PC, PC / DAG, PC / Chol or PC / Toc) and HED were mixed in chloroform. After evaporating the solvent and drying under vacuum

overnight, the mixture was dissolved in Tris-HCl buffer to give mixed micellar solutions.

Hydrolysis Reaction of SUV by PLD. PLD catalyzes the hydrolysis of PC in SUV with the formation of PA and choline. The choline concentration was monitored using a choline oxidase - oxygen electrode. The initial velocity was determined from the slope of the choline production within 5 min. The dissociation of PLD SUV complexes was separately estimated by the ultrafiltration method.

Ultrafiltration PLD was incubated with SUV in Tris-HCl buffer. Free PLD in the incubation medium was separated from SUV-associated PLD by ultrafiltration. The ultrafiltration membrane used was rated at 100,000 MW cutoff (YM100 DIAFLO ultrafilters, W.R.Grace & Co.-Conn.). The operating pressure was about 10 psi. The free PLD amount in the filtrate was determined by measurement of the enzyme activity in a standard LPC micellar solution.

FT-IR Spectroscopy. Samples for infrared spectroscopic analysis were prepared in 50μm thick cells with CaF_2 windows. The infrared spectra were measured with a Nicolet 205 Fourier transform infrared (FT-IR) spectrometer equipped with an Hg-Cd-Te detector. The sample temperature was controlled by means of a block assembly through which water circulated and was monitored by a thermosensor placed at the edge of the cell window. The 256 interferograms collected were analyzed using Nicolet SX software on a 620 workstation. The resolution was 4 cm^{-1}. The subtraction of spectra in buffer was carried out to remove the contribution of water bands. The accuracy of the frequency reading was better than ± 0.1 cm^{-1}.

Results

Retardation of Choline Production Figure 2-1 shows the time courses of choline production in 1 and 10 mM PC SUV in Tris-HCl buffer. The

enzymatic hydrolysis velocity of PC declined progressively and was 5 ~ 6 % of the initial value after a reaction period of 30 min. The reaction velocity was retarded after conversion of about 1.5 % of PC SUV (corresponding to about 3 % of PC at the outer leaflets of SUV) to PA, irrespective of the SUV and Ca^{2+} concentrations. Adding fresh substrate (PC SUV) to the reaction mixture during the retardation phase induced abrupt choline production, also following the retardation phase after conversion of about 3 % PC at outer leaflets of the fresh SUV (Fig. 2-1). These results demonstrated that the enzyme itself is not inactive at retardation phase, but has a high level of activity only for fresh substrates.

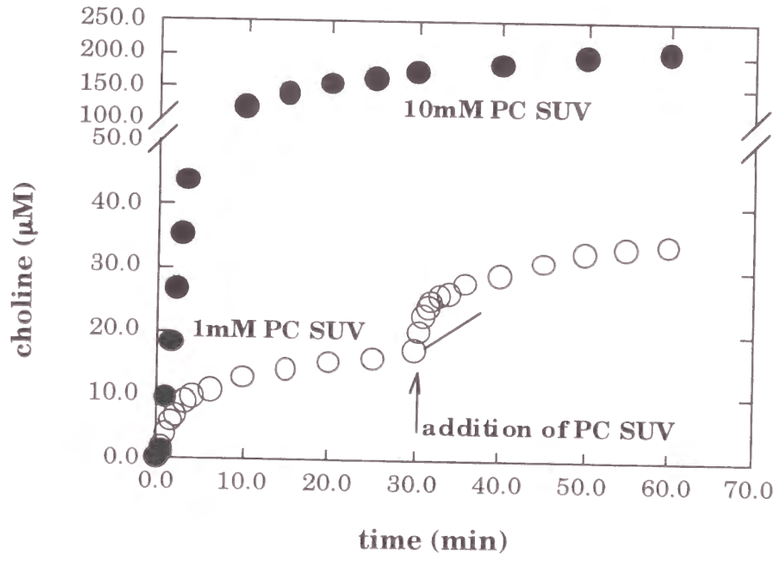


Fig. 2-1 Reaction curves for the hydrolysis of PC SUV by PLD at pH 8.0 and 30°C.

The arrow shows the addition of fresh substrate (1mM PC SUV) to the reaction mixture. The solid line was evaluated on the assumption that PLD competitively binds to fresh substrates and postsubstrates (see chap. 3).

Adding more Ca^{2+} (~ 0.3 mM) or choline did not significantly influence the retardation (data not shown). The initial high velocity was not

recovered by readdition Ca^{2+} after it was eliminated from the reaction mixture (45-min incubation with 100 μM EDTA in a reaction mixture in the retardation phase, followed by adding 150 μM Ca^{2+} to reactivate PLD). The dissociation coefficient of the PLD - PC SUV complex was estimated by the ultrafiltration method as a function of reaction time. Table 2-1 shows that the coefficient was 0.64 mM at the initial stage of high PLD activity (~5 min), and the affinity of PLD to PC SUV was increased markedly at the retardation phase, indicating that most of the enzyme bound to PC SUV. The dissociation coefficient at the retardation phase, 0.17 mM, was close to that of the PLD - mixed PC / PA SUV complex (including over 80 mole% PA). These results suggested that PA molecules at the vesicle surface are involved in the high affinity of PLD for SUV and the retardation of hydrolysis.

Table 2-1 Dissociation coefficient of PLD to PC/PA SUV at 30°C

PC / PA in SUV	Dissociation coefficient [mM]	
(mole ratio)	< 5 min	> 60 min
10 / 0	0.64 ± 0.1	0.17 ± 0.05
5 / 5	0.53 ± 0.05	–
3 / 7	0.26 ± 0.03	–
2 / 8	0.20 ± 0.03	–
1 / 9	0.21 ± 0.02	–
0 / 10	0.20 ± 0.03	0.13 ± 0.02

Values are represented as means ±S.D. of two or three independent experiments.

Figure 2-1 shows about 1.5 % (about 3% at the outer leaflet) conversion of PC to PA after 30 min irrespective of PC SUV concentration. Here, the PLD concentration was about 40 nM, whereas the vesicle (particle) concentration was 300 to 3000 nM. Therefore, the enzyme was exchanged among substrate particles, each particle was equally hydrolyzed,

and the hydrolysis product, about 1.5 mol% of PA, was responsible for the diminished activity of the enzyme.

SUV of mixed PC and PA (molar ratio: 9.5 / 0.5) were examined (Fig.2-2). The homogeneously dispersed PA in SUV [49] did not affect the time course of choline production. Addition of the nonionic surfactant HED to destroy the lipid membrane structure brought about abrupt choline production without the retardation of PC hydrolysis. This figure also shows that PC molecules previously solubilized in nonionic micelles, HED micelles (PC : HED = 1 : 10), were hydrolyzed about 4 times faster than those in SUV. Furthermore, the enzymatic reaction was not retarded and all substrates in the micelles were hydrolyzed. These observations indicated that the hydrolysis product, PA, and the structure organized by the nascent PA at the surface of substrate vesicles participate in the retardation phase.

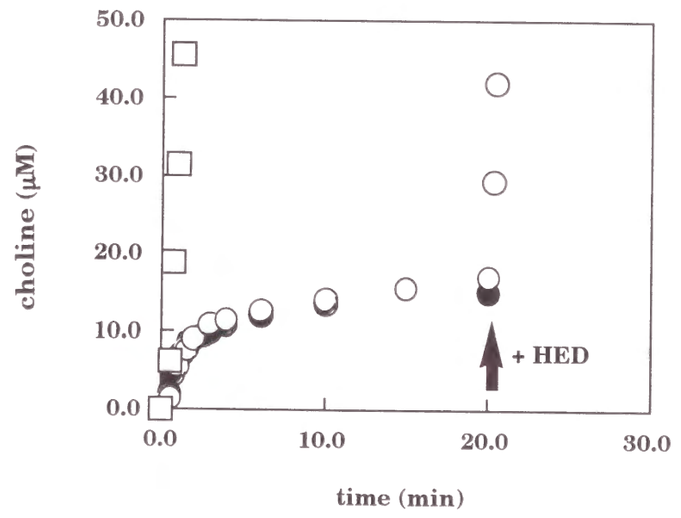


Fig. 2-2 Reaction progress curves of PC hydrolysis by PLD for various substrate preparations. The arrow shows the addition of HED (10mM) to the reaction mixture.

○, 1 mM PC SUV; ●, PC/PA SUV (PC:0.95mM, PA: 0.05 mM);

□, solubilized PC in HED micelles (PC:1mM, HED:10mM)

Hydrolysis of PC in SUV Containing Neutral Lipid. The enzymatic hydrolysis of PC increased with addition of neutral lipids, Chol, DAG or Toc to the PC bilayers (PC SUV) .

Figure 2-3 shows the effects of DAG on the choline production in SUV. The amount of choline produced in PC SUV (2mM) was 45 μM over a period of 30 min (control). Increases in the mol% of DAG led to the enhanced initial velocity and reduced the retardation of PC hydrolysis (see the last column in Table 2-1). The addition of more than 20 mol% of DAG disintegrated the bilayer structure and induced formation of non-bilayer structures; hexagonal and/or cubic phases of inverse topology [50, 51]. The inversed phase separated from the reaction medium.

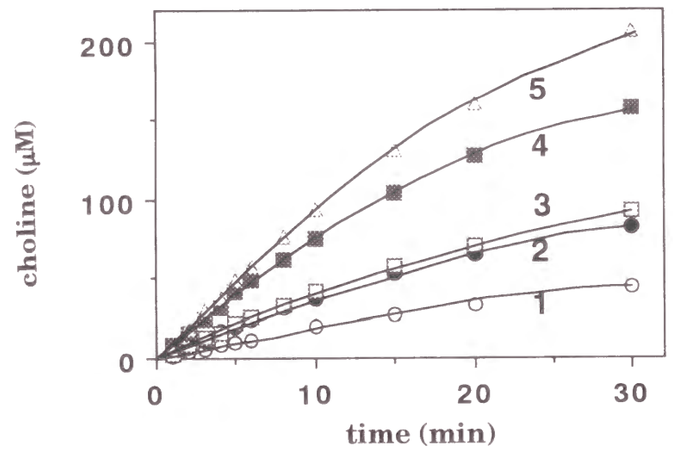


Fig. 2-3 Reaction progress curves of PC hydrolysis by PLD for PC/DAG SUV.

The PC concentration in SUV was 2mM.

DAG (in mol%), 1, 0; 2, 6.3; 3, 9.3; 4, 14.8; 5, 18.3.

Kinetics of PLD Hydrolysis. The reaction velocity, $\boldsymbol{v}$, was determined from the initial slope of the enzymatic production of choline (5min) with changes initial substrate concentration, $\boldsymbol{S}$, (in mM of PC in SUV) at a fixed

neutral lipid composition. The double-reciprocal plots, $1/\boldsymbol{v}$ vs. $1/\boldsymbol{S}$ were all linear. Similar correlations were observed for the reaction of phospholipase A_2 in PC-SUVs [35]. The Lineweaver - Burk plots gave the Vmax(app) (μM / min) and Km(app) (mM) values shown in Table 2-2. Here, Vmax(app) and Km(app) are apparent maximum velocity and apparent Michaelis constant for PC hydrolysis, respectively, as described in chapter 1.

Table 2-2 The Km(app) and Vmax(app) values in the hydrolysis of PC by PLD

Degradation ratio at 30 min is represented as mol% at the outer leaflet of PC SUV. Calcium concentration in Tris-HCl buffer was 7.8 μM.

Bilayers	Km (app) (mM)		Vmax (app) (μM/min)		degradation ratio at 30 min
PC-SUV	0.844 ± 0.137		3.05 ± 0.32		3 %
DAG/PC-SUV					
DAG 6.3%	0.851 ± 0.229	⇨	5.40 ± 0.85	⇧	
14.8%	1.19 ± 0.420		12.7 ± 2.8		
18.3%	1.15 ± 0.420		16.8 ± 4.1		20 %
Chol/PC-SUV					
Chol 18.3%	1.30 ± 0.590	⇧	5.87 ± 0.97	⇧	3 %
26.0%	2.37 ± 1.40		8.51 ± 4.29		
35.0%	1.73 ± 3.30	⇩	6.48 ± 0.85	⇩	
47.0%	1.30 ± 1.10		5.09 ± 0.24		
Toc/PC-SUV					
Toc 18.0%	0.871 ± 0.229	⇨	7.43 ± 1.55	⇧	
24.0%	0.796 ± 0.201		14.9 ± 1.90		10 %

DAG and Toc exerted similar effects on the PC hydrolysis by PLD. The Km(app) and Vmax(app) values in PC SUV were 0.844 mM and 3.05 μM / min, respectively. The Vmax(app) value increased significantly with the mol% of the neutral lipid in both PC / DAG and PC / Toc SUV. Furthermore, degradation ratio in the retardation phase (at 30 min) was markedly increased by incorporating these neutral lipids, indicating relaxation of the retardation phase. The effect of Toc, however, was half of that of DAG. The Km(app) value was virtually independent of the DAG or Toc composition. These results suggested that the incorporation of DAG

and Toc increased the catalytic activity of PLD on the SUV surface, but did not change the binding affinity of the enzyme to the surface.

In the Chol-containing SUV, both Km(app) and Vmax(app) increased with mol% of Chol and compensated each other in the degradation of PC.

The enzymatic hydrolysis of PC was also examined in HED micelles (PC / HED = 1 / 10 molar ratio). The concentration of HED was sufficient to largely preclude interactions between PC molecules in the micelles. The Km(app) value was smaller than those in SUV by one order of magnitude. In the HED micelles, the addition of DAG, Toc or Chol (up to 10 mol% of PC) did not influence the kinetic parameters of PC hydrolysis by PLD (data not shown).

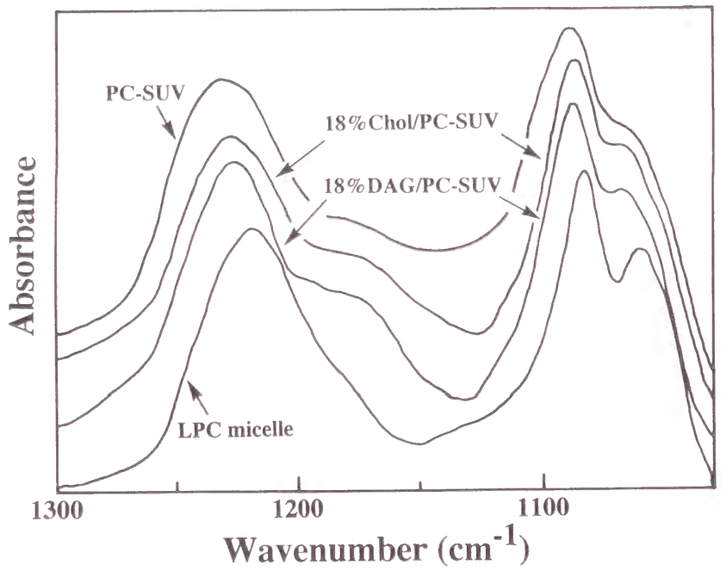


Fig. 2-4 Infrared spectra of PC vesicles and LPC micelles for the PO_2^- double bond stretching region at 30°C.

FT-IR Spectra for Antisymmetric PO_2^- Stretching Region of PC. Figure 2-4 shows infrared spectra of PC SUV, PC / 18.3 mol% DAG SUV, PC / 18.3 mol% Chol SUV and LPC micellar solution for the PO_2^- double bond stretching bands (1000 - 1300cm^{-1}) at 7.8 μM Ca^{2+}. Spectra were assigned according to ref. [52]. In PC SUV, the symmetric and

antisymmetric stretching modes had maximum frequencies at 1088.4cm^{-1} and 1231.7cm^{-1}, respectively. These values were consistent with those of dipalmitoylphosphatidylcholine vesicles in the liquid crystalline state [53]. Incorporation of DAG or Chol into PC SUV shifted the PO_2^- antisymmetric stretching band (ν_{max}) to a lower frequency. The bandwidth appeared to slightly decrease. However, accurate estimation of the bandwidth was difficult because of overlap with the C-O stretching band of the ester group at appox. 1170cm^{-1} [52]. In LPC micellar solution, the PO_2^- antisymmetric stretching band was observed at 1220.6cm^{-1}. Addition of neutral lipids to PC SUV led to a decrease in the frequency. Incorporation of 18.3 mol% DAG into PC SUV brought about a 5 cm^{-1} shift to a lower frequency. A marked downshift of about 11cm^{-1} (from PC SUV) was found for the LPC micellar solution.

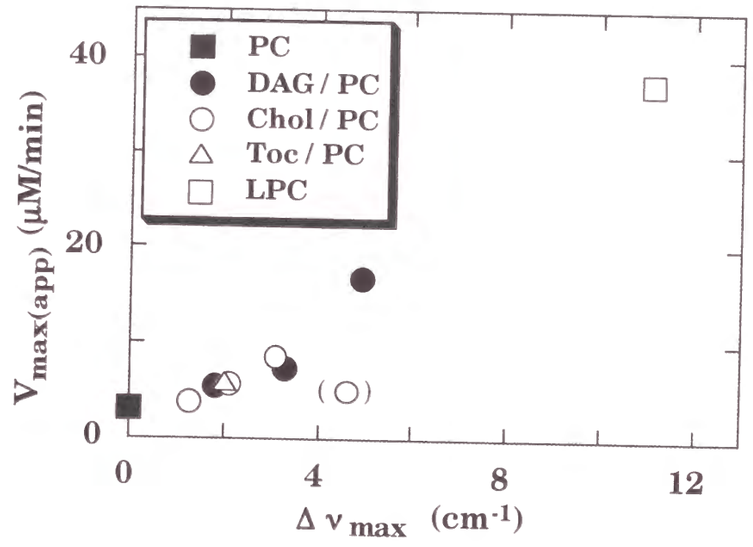


Fig. 2-5 Relationship between the Vmax(app) value and the Δνmax.

Figure 2-5 shows the correlation between $\Delta\nu_{max}$ and Vmax(app), where $\Delta\nu_{max}$ is the shift of the antisymmetric PO_2^- band for PC in mixed SUV or LPC in micelles from that in PC-SUV. These observations indicated that

the catalytic activity of PLD increased with the downshift of the antisymmetric stretching band of PC except PC / 47.0 mol% Chol SUV.

On the other hand, Ca^{2+} did not lead to any detectable frequency shift of PO_2^- in the concentration range used for the assay of the enzymatic reaction (0 - 50 μM) (data not shown).

Discussion

Effects of Neutral Lipids in SUV on the K_m(app) Value. The incorporation of DAG, Chol or Toc molecules into SUV influenced the kinetic parameters of PC hydrolysis by PLD (Table 2-1), while neutral lipids in the HED micelle did not have any significant effects. Thus, the effects of these lipids were not due to specific interactions with the enzyme itself, but rather upon the organization of substrate when it was dispersed in bilayer form. The indirect activation of PLD by Toc and DAG contrasted with the activation of cobra venom phospholipase A_2 toward phosphatidylethanolamine by phosphatidylcholine in mixed micelles where direct interactions between the enzyme and the activator were shown to play important roles[54, 55].

The addition of DAG, Chol and Toc showed different effects on the K_m(app) value. The DAG molecules have an orientation similar to that of the PC molecules in bilayers. The acyl chains of DAG are roughly parallel to those of PC. The glycerol backbone of DAG is approximately parallel to the acyl chains and the sn-2 carbonyl group is closer to water-phase than the sn-1 carbonyl group [56]. DAG used in this study was derived from egg yolk phosphatidylcholine (PC) and had the same fatty acid composition as PC. Therefore, the effect of DAG on the fluidity of bilayers may be much smaller than those of Chol. The near constant values of Km(app) indicated that the hydrophobic interaction between PLD and SUV was little influenced by the addition of DAG.

The K_m(app) value was also nearly constant with the addition of Toc to PC SUV. Spreading pressure of PC and Toc mixture varied with the mole fraction of Toc (data not shown), suggesting complete miscibility of PC and

Toc in the bilayer and hexagonal phase, and strong attractive interactions between Toc and PC [57 - 62]. FT-IR and ESR measurements indicated that the incorporation of Toc broadens the gel to liquid-crystalline phase transition of L -α-dipalmitoylphosphatidylcholine (DPPC) bilayers [47, 63]. Furthermore, NMR studies have shown that the hydrocarbon chain of Toc is packed closely with the DPPC molecule [47]. The ordering effects of Toc on PC bilayers are, however, much smaller than those of Chol [64 - 66]. The addition of Toc did not influence the binding of PLD to the bilayers unlike the addition of Chol.

The incorporation of Chol into SUV lowers gauche rotamer formation of the acyl chain of PC and enhances the order in bilayers [56, 66]. The penetration of hydrophobic moieties of PLD into hydrophobic regions of the ordered bilayers is inhibited and the PLD affinity may decrease (i.e. increase in K_m(app) value). Similar effects of Chol have been observed in the interactions between bilayers and plasma proteins [67 - 69]. Physicochemical studies have identified PC-Chol mixed bilayers as liquid - disordered phase in the Chol composition range from 0 to 23 mol% and liquid-ordered phase in the range from 25-33 to 50 mol% [56,66 and 70]. The phase change and phase separation probably cause the return of the kinetic parameters in SUVs with 35 and 47 mol% Chol (Table 2-1).

Susceptibility of P-O Bond of PC and LPC to PLD and Effects of Neutral Lipids on the V_{max}(app) Value. The asymmetric stretching band for the PO_2^- double bond of PC is useful to monitor the hydration state of the polar head group [71]. A frequency of approx. 1220 cm^{-1} characterizes a fully hydrated PO_2^- group, whereas dehydration makes it appear at a higher frequency (approx. 1240 cm^{-1}) [72]. The shift to lower frequency by incorporation of neutral lipids (Fig. 2-4) is therefore caused by enhanced hydration of PO_2^- group of PC at the membrane surface. In phosphatidylcholine bilayers, the P^--N^+ dipoles align parallel to the plane of membrane surface and the unesterified phosphate oxygens form hydrogen bonds with adjacent molecules through water molecules [73]. The rather small changes in ^{31}P chemical shift anisotropy of PC bilayers demonstrated that the average orientation and motion of the head group

segments are not significantly affected by incorporation of DAG (< 20 mol%) [64] or Chol (< 50 mol%)[74]. ^{13}C-NMR spectroscopy also showed that the DAG molecules have an orientation similar to that of the PC molecules in bilayers and that the glycerol backbone of DAG is approximately parallel to the acyl chains [64]. On the other hand, chemical shift anisotropy measurements have indicated that the conformation of the polar head group in PC bilayers is slightly perturbed by the incorporation of Toc(< 30 mol%) [63]. ^{13}C-NMR studies showed that the spin-lattice relaxation time for the choline moiety is increased by incorporation of Toc into PC bilayers[44]. On the basis of the deuterium quadrupolar splitting of choline methylene groups of PC, Brown and Seelig reported that Chol in PC bilayers acts as a spacer molecule in the polar head group region, increasing the separation between head groups [74]. NMR studies showed a reduction of the ^{31}P spectral width, consistent with increased motional freedom of the phosphate [75]. Addition of Chol (up to 26.0 mol%), DAG (up to 18.3 mol%) and Toc (up to 24 mol%) thus increased the space around the P-O bond and enhanced the hydration of PO_2^- (i.e. shift of antisymmetric PO_2^- band to a lower frequency). The ν_{max} value further decreased with increasing Chol content to 47.0 mol% (Table 2-2). Thermodynamic analysis studies of PC-Chol mixtures have shown phase changes and phase separation around the Chol mol% of 25-30 [56, 76].

In the present study, addition of DAG, Chol and Toc to PC SUV increased the Vmax(app) value of PLD. The Vmax(app) value ($= f \cdot E_0 \cdot k_2$) is associated with the degree of activation of PC-PLD complex in the SUV surface (i.e. f) and the decomposition rate of the activated complex to choline, phosphatidic acid and PLD (k_2). The increase in Vmax(app) value was correlated with the downshift of the asymmetric PO_2^- band ($\Delta\nu_{max}$), which was caused by increasing hydration of PO_2^- group of PC (Fig.2-5). The increasing hydration implies increased space around the P-O bond and enhanced motional freedom of the phosphate. As a result, incorporation of the neutral lipids enhanced accessibility of the catalytic site of PLD to the susceptible bond and/or promotes the release of the choline group by incorporating neutral lipids into PC SUV. Similar effects of phospholipid

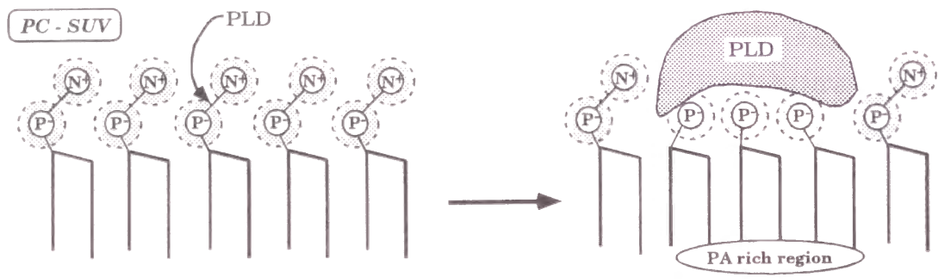
packing on hydrolysis activity of phospholipase A have been demonstrated in lipid monolayers [77, 78]. 1,2-Diacylglycerol has been shown to be an inducer of PLD in several cell types [39, 40]. Coorssen and Rand showed that Chol reduced the amount of DAG required to perturb the bilayer structure of PC [79]. Further studies of the effects of DAG in PC-bilayers containing Chol are required in connection with PLD activation in biological membranes.

In LPC micelles, the hydrolysis by PLD was about twelve fold (Vmax(app) : 37.4 ± 7.0 μM/min) larger than that in PC-SUV. The ν_{max}, 1220.6 cm^{-1}, was consistent with the frequency that characterizes a fully hydrated PO_2^- group[72]. LPC micelles have larger curvature than SUV. NMR studies have shown that the LPC head group moves much more freely than that of diacyl phospholipids [80]. The majority of this increase in motion is attributed to rapid spinning around the sn-1 to sn-2 carbon-carbon bond of the glycerol backbone, which occurs only in the LPC and not in the diacyl-phosphatidylcholine molecules [81]. In addition, LPC probably has a larger head group area at the surface due to the additional hydroxy group [81]. These factors in LPC micelles lead to the extensive hydration and higher susceptibility to PLD.

Product-Retardation of Enzymatic Hydrolysis. PLD molecules bound to PC SUV diffuse laterally at the surface and hydrolyze closely neighboring PC molecules to form small clusters of PA. According to Gouy's theory, if the binding of PLD to SUV is caused by electrostatic interaction it would be roughly proportional to the surface charge density [82]. The nonlinear variation of the dissociation coefficient with the PA fraction (Table 2-1) suggested that the interaction was not simply electrostatic, but rather specific. The enzyme entrapped at the PA cluster on the vesicle surface is inactive to substrate molecules on the same vesicles because of depression of lateral diffusion.

The formation of PA clusters and the retarded hydrolysis seem to depend on the bilayer - organization of PC molecules. The effects of neutral lipid incorporation into the PC bilayer are illustrated in Fig. 2-6. Addition of neutral lipids into PC SUV has two effects. The first is an increased initial

velocity as a result of the increased space around the P-O bond and the enhanced accessibility of the catalytic site of PLD to the susceptible bond. The second effect is the relaxation of product retardation. Neutral lipids intercalated between regularly arrayed phospholipid molecules at the vesicle surface interfered with the cluster formation of PA molecules produced by PLD hydrolysis. Surfactant (HED) molecules added to vesicles in the retarded phase of PLD reaction caused disintegration of the bilayer structure and eliminated the retardation of hydrolysis (Fig.2-2). Here, the SUV in the retarded stage of PLD reaction is designated as "postsubstrate". Postsubstrates are vesicles, which strongly entrap PLD at the PA cluster and lack susceptibility to PLD.



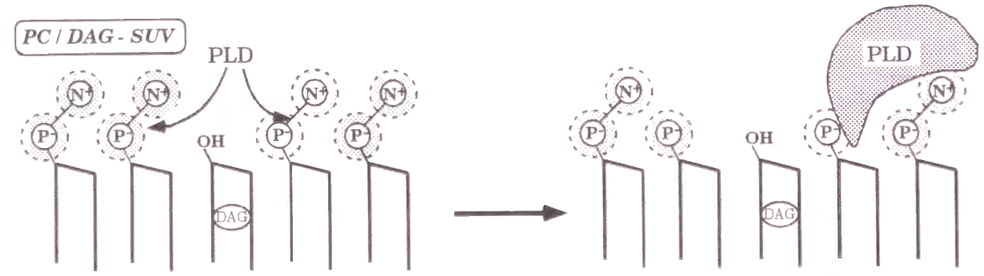


Fig. 2-6 Illustration of the effects of incorporation of neutral lipids into the PC bilayer.

Interactions between PLD and closely neighboring PA molecules may be responsible for retaining the cluster at the SUV surface. In this study, however, the number of PLD molecules in the reaction mixture was too small to interact with all SUV particles simultaneously. The elimination

of Ca^{2+} by EDTA in postsubstrates did not recover the susceptibility to PLD. Therefore, neither PLD nor Ca^{2+} directly participated in cluster formation. PA analogs such as alkylphosphates form acid-soap clusters at pH 8.0, and also associate into separated patches from other lipid components in monolayers by hydrogen bonding [83 - 86]. Such interactions are likely to lead to the local clustering of nascent PA molecules at the SUV surface. The formation and physiological role of local patches of PA in membrane have been suggested by Liscovitch and Cantley in a description of PLD activation by a low molecular weight GTP-binding protein ADP-ribosylation factor (ARF) [49]. Products of phospholipase A_2 (fatty acid) were also reported separate from the substrate, PC, and form clusters in vesicles [87]. Burack et al. reported that only 3% of the hydrolysis product by PLA_2 in large unilamellar vesicles of dipalmitoylphosphatidylcholine produces clusters that entrap the enzyme [88]. Therefore, various physical and chemical properties of the membrane such as lipid packing [51,89], lateral lipid distribution and phase separation [87, 90 - 92] influence phospholipase activities.

Chapter 3
Enhanced Activity of PLD - SUV Complexes

Binding of PLD to substrate SUV is a critical step in interfacial catalysis. This step regulates the magnitude of the interfacial activity of phospholipases [93]. When PLD is weakly bound to SUV and exchanged among SUV rapidly through aqueous solution, the binding and desorption (the on- and off-steps) of the enzyme are involved in each catalytic turnover cycle (PLD in the hopping mode), resulting in reduced catalytic activity. On the other hand, PLD is bound to the SUV surface very strongly and is not desorbed from the surface during the catalytic turnover cycle (PLD in the scooting mode), enhanced enzymatic activity may be observed. When PLD is directly translocated from SUV to SUV by collision between vesicles, during the residential period of the enzyme at the surface, the on- and off-steps are practically eliminated.

The last chapter described the effects of PA SUV and postsubstrate, which have high affinity to and prolonged residential time of PLD, on the interfacial activity of the enzyme. The results indicated critical roles of distribution, localization and translocation of PLD in interfacial hydrolysis.

Materials and Methods

Materials. Egg yolk phosphatidylcholine (PC) was provided by Asahi Kasei Co. (Tokyo). The purity (over 99%) was determined by thin-layer chromatography (TLC). Egg yolk phosphatidic acid (PA) and egg yolk lysophosphatidylcholine (LPC) obtained from Sigma Chemicals Co. (St. Louis, MO) were resolved as single spots on TLC, respectively. Heptaethyleneglycol dodecylether (HED) , a nonionic surfactant, was obtained from Nikko Chemicals (Tokyo; purity above 99 %). All other chemicals obtained from Wako Pure Chemicals (Osaka), were of special grade. Buffer was prepared with water distilled twice from a glass still. PLD obtained from Sigma was as described in the first chapter.

Preparation of Vesicles. PC, PA and mixtures of both were dissolved in chloroform. The solvent was evaporated under reduced pressure and the residual film was dried *in vacuo* overnight to ensure complete solvent removal. The lipid film was hydrated with a buffer containing 10 mM Tris, 150 mM NaCl and 50 μM Ca^{2+} (pH 8.0) unless otherwise noted. The lipid suspension was dispersed by vortex mixing and sonication for 30 min under a nitrogen stream at 5°C using a UD-200 probe-type sonicator (Tomy Seiko) .

Hydrolysis Reaction of SUV by PLD. PLD catalyzes the hydrolysis of PC in SUV with the formation of PA and choline. The choline concentration was monitored using a choline oxidase - oxygen electrode. The initial velocity was determined from the slope of the choline production within 5 min. The dissociation of PLD SUV complexes was separately estimated by the ultrafiltration method.

Results

Production of Postsubstrate. As described in the second chapter, after rapid conversion of a specific amount of PC to PA, PC SUV did not serve as an effective substrate although most enzyme bound to the SUV membrane. SUV with low susceptibility to PLD was termed "postsubstrate" as described in the second chapter. Postsubstrate was characterized by their high affinity and low susceptibility of PC to PLD. When fresh SUV was added to the postsubstrate solution, choline production was unexpectedly higher than that predicted by the competitive inhibition of the postsubstrates shown in Fig.2-1.

Competitive Binding of PLD to Vesicles. The competitive binding effects of PA SUV on the enzymatic hydrolysis of PC and PC / PA (5 / 5) mixed SUV were investigated (Fig. 3-1). About 95% of PLD bound to PA SUV at a concentration of 2mM, because the vesicles have a high binding affinity for PLD with a dissociation coefficient of 0.13 mM.

For the competitive binding of PLD to the substrate and PA vesicles, the initial velocity is presented as

$$v = \frac{V\max(app)\cdot S}{(1+I/K_I)Km(app)+S} \qquad (3\text{-}1)$$

Here, the parameters, Vmax(app) and Km(app) were obtained from the kinetic analysis without PA SUV, and the dissociation coefficient of PLD and PA SUV, K_I, was obtained by a separate binding experiment (the ultrafiltration method). I is the concentration of PA SUV.

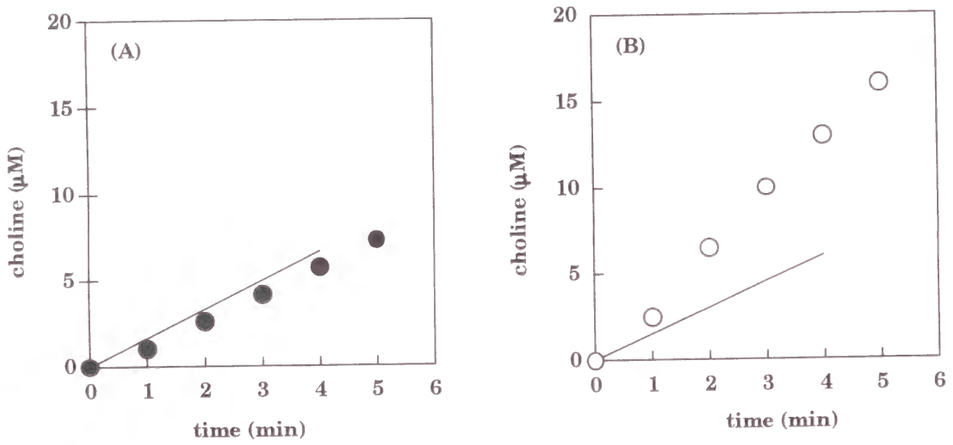


Fig. 3-1 Competition of Substrate SUV with Inhibitor SUV (2mM PA SUV).

Substrate : (A); PC / PA (5 / 5) SUV, (B); PC SUV

Fig. 3-1A shows the time course of choline production in PC / PA SUV by PLD - PA SUV complex preincubated for 1 hr. The solid line in Fig. 3-1A represents the choline production calculated by the competitive inhibition equation (eq. 3-1). The agreement between the experimental and calculated values suggested that the PLD enzyme was competitively distributed among the substrates, PC / PA SUV and the inhibitors, PA SUV throughout the aqueous phase.

When PC / PA mixed SUV were replaced by PC SUV as substrate, PA vesicles did not function as effective competitive inhibitors, giving a several-fold higher PLD activity than that predicted by eq.(3-1), as shown in Fig. 3-1B. Similar results were obtained when fresh PC SUV was added to the reaction mixture in the retardation phase (Fig. 2-1).

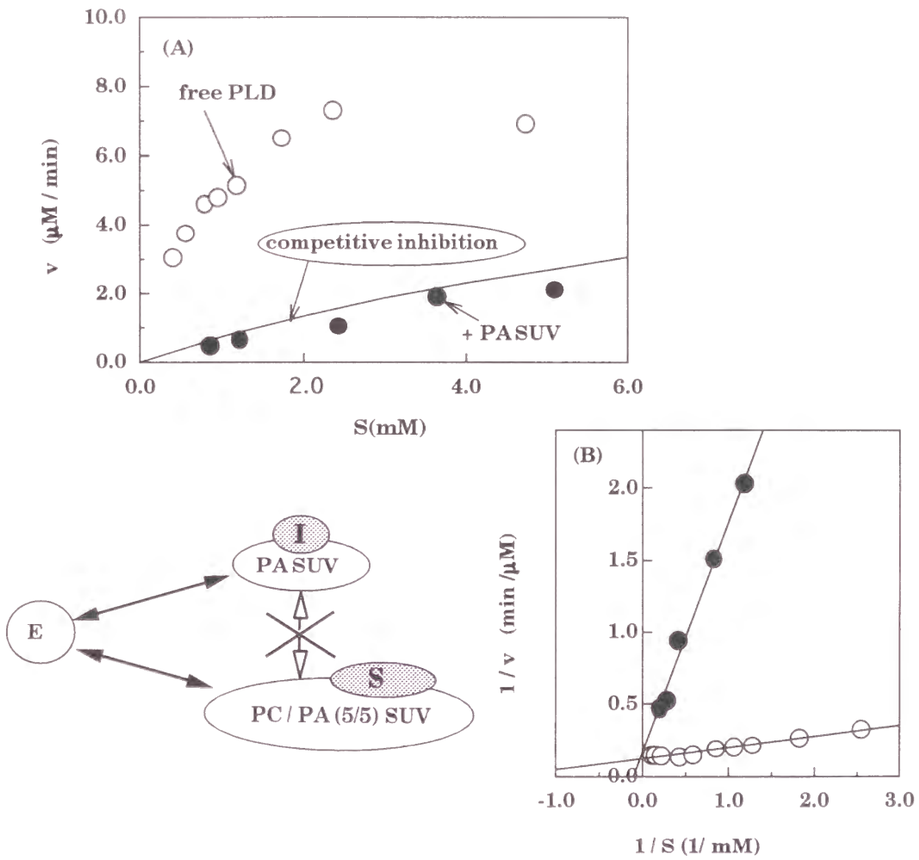


Fig. 3-2 Dependence of initial velocity of PC hydrolysis on the vesicle concentration (A), and Lineweaver-Burk plots (B).

The substrate was PC/PA (5/5) SUV and the reaction was initiated by adding PLD(○) or the enzyme pre-equilibrated with PA SUV for 1 hr (●)

The linear plots (B) gave Vmax(app) and Km(app) as shown in Table 3-1.

The solid curve in figure (A) was calculated using eq. 3-1.

Effects of PLD-SUV Complexes on the Hydrolysis Reaction. The competitive inhibitory effects of postsubstrates and PA SUV on the

enzymatic hydrolysis of substrate SUV, PC or PC / PA mixed SUV were examined. PA SUV and postsubstrates had high affinity for PLD, with dissociation coefficients of 0.13 ~ 0.17 mM and negligible susceptibility to PLD reaction. Figure 3-2A shows the ***S*** - ***v*** profiles for hydrolysis of the negatively charged SUV (PC/PA = 5 / 5) by PLD in the presence and absence of 2 mM PA SUV. The presence of PA SUV in the reaction mixture markedly decreased the hydrolysis velocity. The double reciprocal ($1/S \sim 1/v$) plots presented in Fig. 3-2B gave similar Vmax(app) values (intercepts to the $1/v$ axis) with and without 2 mM PA SUV (Table 3-1).

Table 3-1 Kinetic parameters obtained for the PC hydrolysis with and without inhibitor SUV.

Substrate SUV	Inhibitor SUV	Vmax(app) [μM / min]		Km(app) [mM]	
		Obseved	Calculated	Obseved	Calculated
PC SUV	- postsubstrates	8.2 ±1.0	–	0.67 ±0.13	–
	+ postsubstrates	24.8 ±7.6	8.2 ±1.0	6.0 ±2.0	8.6 ±3.0
PC/PA SUV	- PA SUV	7.6 ±1.0	–	0.56 ±0.10	–
	+ PA SUV	7.3 ±0.58	7.6 ±1.0	12 ±4.4	9.2 ±3.7

Inhibitor SUV (postsubstrates or PA SUV) was incubated with PLD for 1 hr and then substrate SUV (PC SUV or PC/PA SUV) was added.
Calculated Km(app) values ($(1+I/K_I)$ Km(app)) were obtained by eq. 3-1.

The solid curve in Fig. 3-2A represents the ***v*** values calculated by eq. (3-1) for hydrolysis of the mixed SUV in the presence of PA SUV. The experimental and calculated values for Vmax(app) and $(1+I/K_I)$Km(app) are compared in Table 3-1. The agreement between the experimental and calculated values suggested that the enzyme was competitively distributed among the substrate SUV and PA SUV throughout the aqueous phase.

As shown in Fig.2-1, addition of fresh substrate to the reaction mixture at the retardation phase (postsubstrates) induced abrupt choline production. The velocity was much higher than that calculated by eq. 3-1 for the simple competitive binding of PLD between postsubstrates and

fresh substrates (solid line in Fig. 2-1). The effects of postsubstrates on the enzymatic reaction of PC SUV are presented in Fig. 3-3A. Incubation of PC SUV with PLD for 1 hr converted the SUV to postsubstrates with high binding affinity and negligible susceptibility to the enzyme.

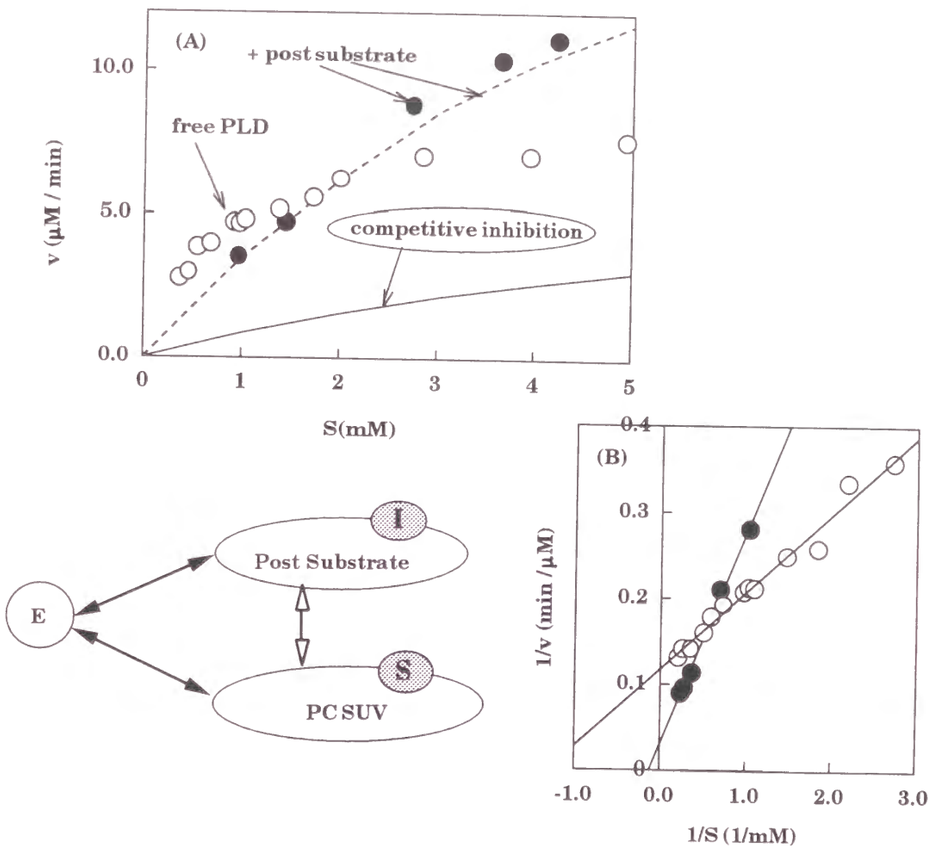


Fig. 3-3 Dependence of initial velocity of PC hydrolysis on the vesicle concentration (A) and the Lineweaver - Burk plots (B).

The substrate was PC SUV, and the reaction was initiated by adding PLD(○) or the enzyme pre-equilibrated with PC SUV for 1 hr beforehand (postsubstrates ●) The linear plots (B) gave Vmax(app) and Km(app) as shown in Table 3-1. The solid curve in figure (A) was calculated using eq. 3-1 for competitive inhibition.
The broken curve in figure (A) was calculated for the enhanced activity of PLD with the kinetic parameters obtained in Fig. 3-3B(●).

Various amounts of fresh substrates (PC SUV) were added to the incubation mixture and the choline production was monitored. The v values calculated by eq. 3-1 for the simple competitive inhibition model (solid line) were much lower than the observed values(Fig. 3-3A). Despite the high affinity of postsubstrates to PLD, they did not competitively inhibit, but rather enhanced the enzymatic activity above the substrate concentration of 2 mM, where the fraction of free PLD in the medium was less than 0.05 of the total PLD. The double reciprocal plot, being linear , gave a $V_{max(app)}$ value of 24.8 μM / min, which was 3-folds larger than that (8.2 μM / min) for the fresh substrates (PC SUV) without postsubstrates (Fig. 3-3B). The observed value of $K_{m(app)}$ was 6.0 mM, which agreed with the calculated value 8.6 mM within experimental error. These results suggested that the postsubstrates brought about an increase in the PLD activity for fresh PC SUV .

Discussion

Competitive Inhibitor and Postsubstrates. PA SUV competitively inhibited the PLD-hydrolysis of PA / PC SUV (Fig. 3-2). Membrane surfaces of both PA SUV and PC / PA SUV have negative net charges because of dissociation of the phosphate group of PA at pH 8.0. The negative surface potential interferes with the direct interaction, collision, between PA SUV and PC / PA SUV. In this system, PLD was transferred between the inhibitor (PA SUV) and substrate vesicles (PA / PC SUV) solely through the aqueous medium, because collision between them was limited by electrostatic repulsion.

On the other hand, postsubstrates, despite a similar high affinity to PLD as PA SUV, did not competitively inhibit the hydrolysis of PC SUV (Fig. 3-3) but rather enhanced the enzymatic activity. The enzyme is entrapped at the PA cluster on postsubstrates , but retains catalytic activity toward fresh PC SUV. Postsubstrates and the substrate vesicles can mutually collide because the electrostatic repulsion is not strong. That is, postsubstrates function as enzyme donors by collision with the substrate

vesicles. The similarly enhanced activity of PLD was also observed for the combination of PC SUV and PA SUV as substrate and competitive inhibitor, respectively (Fig. 3-1B). Here, the collision between the substrates and inhibitors was not suppressed by the electrical repulsion force.

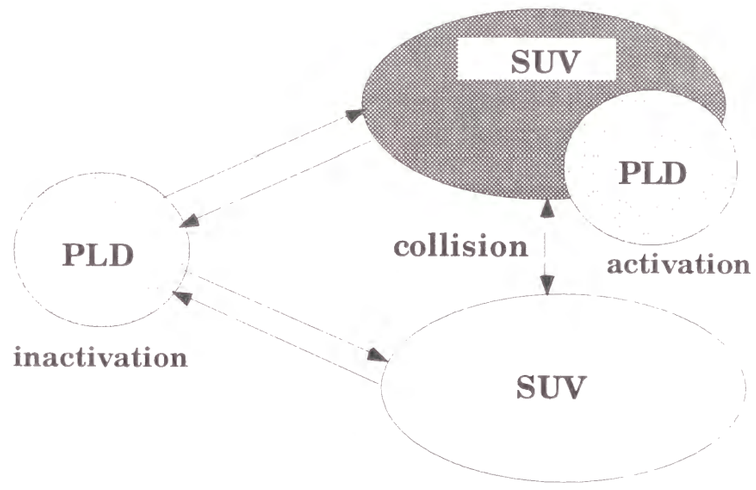


Fig. 3-4 Translocation or exchange of PLD among aqueous medium, substrate SUV (white) and postsubstrates or PA SUV (shadow).
PLD is activated at the SUV surface, and collision of vesicles brings about direct translocation of activated PLD.

PLD on postsubstrates had higher catalytic activity ($V_{max(app)}$ = 24.8 μM / min) than the enzyme transferred through aqueous medium ($V_{max(app)}$ = 8.2 μM / min). When the enzyme was exchanged among vesicles through aqueous medium, conformational changes of the protein at the vesicle surface would be required for the activation. The lag in enzymatic reaction due to the conformational rearrangement could be significant during residence at the SUV surface. The enzyme exchange between vesicles through the aqueous phase would involve an on- and off-step in each cycle, resulting in reduced catalytic activity. Jain et al. [93] have suggested that similar on- and off- steps play critical roles in the

distinct interfacial activation between phospholipase A_2 and prophospholipase A_2. On the other hand, the enzyme attached to postsubstrates or PA SUV encounters substrate vesicles (PC SUV) primarily by collision, and the minimum conformational rearrangement for the activation at the vesicle surface may give a higher V_{max}(app) value than those of PLD from the aqueous phase (Table 3-1). Gong et al. also reported that 1-palmitoyl-2-oleoylphosphatidic acid vesicles lead to a two fold higher activity of PLD toward monomeric short chain-phosphatidylcholine substrates [34].

Conclusions

Phospholipase D from *Streptomyces chromofuscus* (PLD) is known to mimic the effects of endogenous enzyme in mammalian cells. Phospholipase D plays key roles in membrane trafficking and regulation of mitosis as well as signal transduction. However, the precise regulatory mechanism remains to be clarified. The results of the present study can be summarized as follows:

(1) PLD was activated at the PC SUV surface with 10 μM Ca^{2+}. The Ca^{2+}-dependence of PLD suggested that the translocation and catalytic activation of the enzyme may be regulated by physiological alteration in cytosolic Ca^{2+} level.

(2) The reaction velocity was retarded after conversion of about 1.5 % PC SUV (corresponding to about 3 % of PC at the outer leaflets of SUV) to PA, irrespective of the SUV and Ca^{2+} concentrations. The dissociation coefficient of PLD-PC SUV complex at the retardation phase, however, showed that most PLD bound to PC SUV. PC molecules solubilized in nonionic HED micelles (PC : HED = 1 : 10), were hydrolyzed about 4-fold faster than those in SUV. Furthermore, the enzymatic reaction in the micelles was not retarded. These results suggested that the organized cluster of PA preferentially entraps PLD molecules. The enzyme entrapped at the cluster was inactive to substrate PC molecules on the same vesicles because of depression of lateral diffusion.

(3) Addition of neutral lipids to PC SUV influenced the kinetic parameters of PC hydrolysis by PLD. The V_{max}(app) value increased with the mol% of DAG, Toc and Chol in SUV. FT-IR spectra showed that the PO_2^- asymmetric stretching band (ν_{max}) of PC was downshifted by incorporation of the neutral lipid into PC SUV, indicating increased hydration of the polar group. These results suggested that incorporation of neutral lipid into PC SUV brought about an increase in space around the

P-O bond and the enhanced accessibility of the catalytic site of PLD to the susceptible bond.

Furthermore, the increased degradation ratio at 30 min in PC hydrolysis by incorporation of DAG or Toc demonstrated relaxation of product retardation. Neutral lipids incorporated between regularly arrayed phospholipid molecules at the vesicle surface may interfere with the cluster formation of PA molecules and augment lateral diffusion of PLD at the SUV surface.

(4) PLD was strongly entrapped on PA SUV or postsubstrates (inhibitor SUV). When the enzyme was exchanged between the substrate SUV and inhibitor SUV through the aqueous phase, competitive inhibition on the PLD reaction was observed. On the other hand, when the enzyme was directly translocated to the substrate SUV by collision, enzymatic activity was enhanced 3-fold. Therefore, distribution, localization and mode of translocation play critical roles in PLD activation. Similar observations have been reported for phospholipase A2 and C in the interfacial enzymatic reaction.

Acknowledgments

The author wishes to express her sincere gratitude to Professor Dr. Tesurou Handa (Graduate School of Pharmaceutical Science, Kyoto University) for his constant guidance in the course of this work. The author is also grateful to Professor Dr. Koichiro Miyajima (Osaka University of Pharmaceutical Sciences).

The author would like to thank Professor Dr. Katsumi Matsuzaki (Graduate School of Biostudies, Kyoto University) for helpful discussions on this work.

The author is also indebted to many individuals for their support and assistance. Special thanks are owed to Ms Misako Nishii, Takako Mazumi, Akiko Konto, Emi Tokuoka, and other members of Department of Physical Chemistry, Faculty of Pharmaceutical Science, Kyoto University.

Finally, I am thankful to my husband for his support and patient understanding through several years of this study.

Izumi Yamamoto

References

1 Roberts, M. F., and Zhou, C, (1996) in *Encyclopedia of Molecular Biology and Molecular Medicine*, pp. 415-432, VCH Publishers, Weinheim, Germany.

2 Cockcroft, S. (1992) *Biochim. Biophys. Acta* **1113**, 135-160.

3 Huang, C. and Cabot, M. C. (1992) *Biochim. Biophys. Acta* **1127**, 242-248.

4 Chalifa, V., Mohn, H. and Liscovich, M. (1990) *J. Biol. Chem.* **265**, 17512-17519.

5 Wang, P., Anthes, J.C., Siegel, M.I. Egan, R.W. and Billah, M.M. (1991) *J. Biol. Chem.* **266**, 14877-14880.

6 Eldar, H., Ben-Av, P., Schmid, U.-S., Livneh, E. and Liscovitch, M. (1993) *J. Biol. Chem.* **268**, 12560-12564.

7 Kusner, D.J., Schomisch, S.J. and Dubyak, G.R. (1993) *J. Biol. Chem.* **268**, 19973-19982.

8 Bowman, E.P., Uhlinger, D.J. and Lambeth, J.D. (1993) *J. Biol. Chem.* **268,** 21509-21512.

9 Exton, J. H. (1997) *Physiol. Rev.* **77,** 303-320

10 Salmon, D. M., and Honeyman, T. W. (1980) *Nature* **284,** 344-347.

11 Murayama, T., and Ui, M.(1987) *J. Biol. Chem.* **262,** 5522-5529.

12 Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) *J. Biol. Chem.* **265,** 12232-12239.

13 Ferguson, J. E., and Hanley, M. R. (1992) *Arch. Biochem. Biophys.* **297,** 388-392.

14 Tijburg, L.B.M., Geelen, M.J.H. and van Golde, L.M.G. (1989) *Biochim. Biophys. Acta* **1004,** 1 - 19.

15 Iwasaki, Y., Nakano, H., and Yamane, T. (1994) *Appl. Microbiol. Biotechnol.* **42,** 290-299.

16 Imamura, S. and Horiuti, Y. (1979) *J. Biochem.* **85,** 79-95.

17 Imamura, F., Horai, T., Mukai, M. Shinkai, K., Sawada, M. and Akedo, H. (1993) *Biochem. Biophys. Res. Commun.* **193,** 497-503.

18 Liscovitch, M. and Amsterdam, A. (1989) *J. Biol. Chem.* **246,** 11762-11767.

19 Kondo, T., Inui, H., Konishi, F. and Inagami, T. (1992) *J. Biol. Chem.* **267,** 23609-23616.

20 Yang, S. F., Freer, S., and Benson, A. A. (1967) *J. Biol. Chem.* **242,** 477-484.

21 Eibl, A., and Kovatchev, S. (1981) *Methods Enzymol.* **197,** 493-499.

22 Geny, B. and Cockcroft, S. (1992) *Biochem. J.* **284,** 531-538.

23 Xie, M. and Dubyak, G.R. (1991) *Biochem. J.* **278,** 81-89.

24 Grynkiewicz, G., Poenie, M. and Tsien, R.T. (1985) *J.Biol. Chem.* **260,** 3440-3450.

25 Bartlett, G.R. (1959) *J.Biol. Chem.* **234,** 466-468.

26 Matsumoto, K., Seijo, H., Karube, I. and Suzuki, S. (1980) *Biotechnol. Bioeng.* **22,** 1071 - 1086.

27 Campanella, L., Tomassetti, M., De Angelis, G., Sammartino, M.P. and Cordatore, M. (1987) *Clin. Chim. Acta* 169, 175 - 182.

28 Campanella, L., Sammartino, M.P., and Tomassetti, M. *Proc. of the 2nd International Meeting on Chemical Sensors,* Bordeaux, 1986, 580 - 583.

29 Yoshihara, Y. and Watanabe, Y. (1990) *Biochem. Biophys. Res. Commun.* **170,** 484-490.

30 Channon, J.Y. and Leslie, C.C. (1990) *J. Biol. Chem.* **265,** 5409-5413.

31 Clark J.D., Milona, N. and Knopf, J.L (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7708-7712.

32 Kim, D.K., Kudo, I. and Inoue, K. (1991) *Biochim. Biophys. Acta* **1083,** 80-88.

33 Jy, W. and Haynes, D.H. (1987) *Biochim. Biophys. Acta* **929,** 88-102.

34 Dong, G., Justin, C., and Mary, F. R. (1998) *J. Biol. Chem.* **273,** 12195 - 12202.

35 Kensil, C. R. and Dennis, E. A. (1979) *J. Biol. Chem.* **254,** 5843 - 5848.

36 Yeagle, P.L. (1988) in *The Biology of Cholesterol* (Yeagle, P.L., ed.) CRC Press, Boca Raton.

37 Dawson, R.M.C., Hemington, N.L. and Irvine, R.F., (1983) *Biochem. Biophys. Res. Commun.* **117,** 196 - 201.

38 Dawson, R. M. C., Irvine, R. F., Bray, J. and Quinn, P. J. (1984) *Biochem. Biophys. Res. Commun.* **125,** 836 - 842.

39 Billah, M. M., Pai, J-K., Mullmann, T. J., Egan, R. W., and Siegel, M.I. (1989) *J. Biol. Chem.* **264,** 9069 - 9076.

40 Liscovitch, M. (1989) *J. Biol. Chem.* **264,** 1450 - 1456.

41 Vajanamarhutue C., Wilairat P., and Komaratat, P. (1979) *J. Nutr* **109,** 848 - 855.

42 Fukuzawa, K., and Uchiyama, M. (1973) *J Nutr Sci Vitaminol* **19,** 433-453

43 Hulstaert, CE., Gijzel, W. P., Hardonk, M. J., Kroon, A. M., and Molenaar, I. (1975) *Lab Invest* **33,** 176-186

44 Cushley, R. J., and Forrest, B. J. (1977) *Can J Chem.* **55,** 220-226

45 Cushley, R. J., Forrest, B. J., Gillis, A., and Tribe, J. (1979) *Can J Chem.* **57,** 458 - 465

46 Stillwell, W., and Bryant, L. (1983) *Biochim Biophys Acta* **731,** 483-486

47 Srivastava, S., Phadke, R. S., Govil, G., and Rao, C. N. R. (1983) *Biochem Biophys Acta* **734,** 353-362

48 Fukuzawa, K., Chida, H., and Suzuki, A. (1980) *J Nutr Sci Vitaminol* 26, 427 - 434.

49 Liscovitch, M., and Cantley, L. C. (1994) *Cell* **77,** 329 - 334

50 Das, S., and Rand, R. P. (1986) *Biochemistry* **25,** 2882 - 2889.

51 Seddon, J. M. (1990) *Biochemistry* **29,** 7997 - 8002.

52 Casal, H. L., and Mantsch, H. H. (1984) *Biochim. Biophys. Acta* **779,** 381-401.

53 Asher, I. M., and Levin, I. W. (1977) *Biochim. Biophys. Acta* **468,** 63-72.

54 Roberts, M. F., Adamich, M., Robson, R. J., and Dennis, E. A. (1979) *Biochemistry* **18,** 3301 - 3308.

55 Adamich, M., Roberts, M. F., and Dennis, E. A. (1979) *Biochemistry* **18,** 3308 - 3314.

56 Sankaram, M.B. and Thompson, T.E. (1990) *Biochemistry* **29,** 10676 - 10684.

57 Nakajima, K., Utsumi, H., Kazama, M., and Hamada, A. (1990) *Chem Pharm Bull* **38,** 1-4

58 Nakagaki, M., Tomita, K., and Handa, T. (1985) *Biochemistry* **24,** 4619-4624

59 Nakagaki. M,, and Funasaki, N. (1974) *Bull Chem Soc Jpn* **47,** 2482-2485

60 Handa, T., and Nakagaki, M. (1979) *Colloid Polym Sci* **257,** 374-381

61 Handa, T., Saito, H., and Miyajima, K. (1990) *Biochemistry* **29,** 2884-2890

62 Defay, R., Prigogine, I., Bellmans, A., and Everett, D. E. (1966) *Surface Tension and Adsorption* 71 - 84 Longmans, Green, London

63 Villalain. J., Aranda, F. J., Gomez-Fernandez, J. C. (1986) *Eur. J. Biochem.* **158,** 141-147

64 Hamilton, J.A., Bhamidipati, S.P., Kodali, D.R. and Small, D.M. (1991) *J. Biol. Chem.* **266,** 1177 - 1186.

65 Wassall, S. R., Thewalt, J. L., Wong, L., Gorrissen, H., and Cushley, R. J. (1986) *Biochemistry* **25,** 319-326

66 Davies, M. A., Schuster, H. F., Brauner, J. W., and Mendelsohn, R. (1990) *Biochemistry* **29,** 4368-4373

67 Untracht, S. H., (1982) *Biochim. Biophys. Acta* **711,** 176-192.

68 Williams, K. J., Werth, V. P., and Wolff, J. A., (1984) *Prospective Biol. Med.* **27,** 417 - 431.

69 Handa, T., Saito, H., Tanaka, I., Kakee, A., Tanaka, K., and Miyajima, K. (1992) *Biochemistry* **31,** 1415 - 1420.

70 Vist, M. R., and Davis, J. H. (1990) *Biochemistry* **29,** 451-464

71 Casal, H.L., Mantsch, H. H. and Hauser, H. (1987) *Biochemistry* **26,** 4408-4416.

72 Fringeli, U.P. and Gunthard (1981) in *Membrane Spectroscopy* (Grell, E., ed.), pp. 270-332, Springer-Verlag, New York.

73 Pearson, R.H. (1979) *Nature* **281,** 499-501.

74 Brown, M.F. and Seelig, J. (1978) *Biochemistry* **17,** 381-384.

75 Guo, W. and Hamilton, J. A. (1995) *Biochemistry* **34,** 14174-14184.

76 Huang, T.-H., Lee, C.W.B., DasGupta, S. K. and Griffin, R.G. (1993) *Biochemistry* **32,** 13277-13287.

77 Shah, D. O., and Schulman, J. H. (1967) *J. Colloid Interface Sci.* **25,** 107- 119.

78 Sen, A., Isac, T. V., and Hui, S. W., (1991) *Biochemistry* **30,** 4516 - 4521.

79 Coorssen, J.R. and Rand, R.P. (1990) *Biochem. Cell Biol.* **68,** 65-69.

80 Allegrini, P.R., van Scharrenburg, G., de Haas, G.H. and Seelig, J. (1983) *Biochim. Biophys. Acta* **731,** 448-455.

81 Stafford, R.E. and Dennis E.A. (1988) *Colloids and Surfaces* **30,** 47-64.

82 Davis, J. T. and Rideal, E. K. (1961) in *Interfacial Phenomena,* Academic Press, New York pp 75 - 107

83 Parreira, H. C. (1965) *J. Colloid Sci.* **20,** 742 - 754.

84 Gershfeld, N. L. and Pak, C. Y. (1967) *J. Colloid Interface Sci.* **23,** 215 - 220.

85 Handa, T. and Nakagaki, M. (1976) *YAKUGAKU ZASSHI* **96,** 912 - 918.

86 Nakagaki, M., Kishimoto, H, and Hirano, K. (1970) *YAKUGAKU ZASSHI* **90,** 1118 - 1125.

87 Burack, W. R., Yuan, Q., and Biltonen, R. L. (1993) *Biochemistry* **32,** 583 - 589.

88 Burack, W. R., Gadd, M. E., and Biltonen, R. L. (1995) *Biochemistry* **34,** 14819 - 14828.

89 Rao N M, Sundaram C S (1993) *Biochemistry* 32: 8547 - 8552

90 Grainger D W, Reichert A, Ringsdorf H, Salesse C (1990)*Biochim. Biophys. Acta* 1023: 365 - 379

91 Reichert A., Ringsdorf H, Wagenknecht A. (1992) *Biochim. Biophys. Acta* 1106: 178 - 188

92 Burack W R, Biltonen RL (1994) *Chem. Phys. Lipids* 73: 209 - 222

93 Jain M K, Roger J, de Haas G H (1988) *Biochim. Biophys. Acta* 940: 51 - 62